Arch Toxicol (1994) 68: 79–84

Archives of
Toxicology

Bacterial mutagenicity of 2-chloro-1,3-butadiene (chloroprene) caused by decomposition products

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Received: 1 August 1993/Accepted: 19 October 1993

Abstract. Since the literature on genotoxicity of 2-chloro-1,3-butadiene (chloroprene) is controversial, the mutagenicity of this compound was reinvestigated with respect to its chemical stability. Because of the volatility of chloroprene, Ames tests with *S. typhimurium* TA 100 were carried out with gas-tight preincubation. Propylene oxide, a volatile direct mutagen, served as a positive control. Benzo[a]pyrene was used as a control for an indirect mutagen. Using this experimental regimen, freshly distilled chloroprene was not mutagenic. However, a mutagenic effect occurred linearly with increasing age of the chloroprene distillates. Aged chloroprene gave the same positive results whether preincubation was gas-tight or not. Analysis by gas chromatography (GC) revealed several decomposition products in aged chloroprene distillates. The direct mutagenicity towards TA 100 correlated with the integrated amounts of four of these substances; these substances always occurred in the same relative ratio. When chloroprene was kept under anaerobic conditions, products occurred with time which were partly different from those obtained under aerobic conditions. The direct mutagenicity of anaerobically aged chloroprene was only weak, but the mutagenic effect was enhanced about two- to threefold by addition of S9 mix. Partial identification of chloroprene decomposition products was done by gas chromatography-mass spectrometry (GC-MS): major byproducts of chloroprene, probably responsible for mutagenic properties of aged chloroprene samples, were cyclic chloroprene dimers.

Key words: Chloroprene – 2-Chloro-1,3-butadiene – Mutagenicity – Ames test

This paper compiles data from a thesis (Westphal, 1993) submitted to the "Mathematisch-Naturwissenschaftliche Fakultät der Heinrich-Heine Universität Düsseldorf", Düsseldorf/Germany, in partial fulfillment of the requirements for the doctoral degree.

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Introduction

Chloroprene (2-chloro-1,3-butadiene) is a volatile, highly reactive monomer for the production of polychloroprenes. Chloroprene latexes are used as a binder in paper, upholstery, asbestos products, etc. (Hargreaves and Thompson 1964). In Western Europe, the USA and Japan the annual chloroprene production was estimated at 400000 tonnes (Weissermel and Arpe 1990).

The chemical structure of chloroprene is similar to those of vinyl chloride, a proven human carcinogen (Creech and Johnson 1974), and of 1,3-butadiene which is clearly carcinogenic in animal experiments (IARC 1987b). This has prompted several authors to investigate the mutagenic and carcinogenic properties of chloroprene, as well as possible mechanisms of action.

Chloroprene was mutagenic in the Ames test with *Salmonella typhimurium* (TA 100, TA 98, TA 1538, TA 1537 and TA 1535; Bartsch et al. 1975, 1979; Willems 1978, 1980). Addition of an metabolic activation system (S9 mix) enhanced the mutagenic effect in a NADPH-dependent way. Spectral analysis of chloroprene, which was passed through a mouse liver microsomal suspension supplemented with 4 (4-*N*-nitrobenzyl)pyridine, suggested the biological formation of a chloroprene epoxide as reactive intermediate (Bartsch et al. 1979).

Chloroprene was positive in a recessive lethal test with *Drosophila melanogaster* (Vogel 1979) and in a micronucleus test with mice (Li and Xue 1986). Chloroprene was not active in an HGPRT test with V79 cells (Drevon and Kuroki 1979), in a micronucleus test with rats (Willems and Immel 1978) and in a dominant lethal test with male albino rats (Immel and Willems 1978a, b). In addition, no cytogenetic effects were observed (Tice et al. 1988; Shelby 1990).

Long-term studies involving chronic exposure of experimental animals to chloroprene were negative (BD-IV rats: Ponomarkov and Tomatis 1980; Wistar SPF rats: Reuzel et al. 1980; Syrian hamsters: Reuzel and Bosland 1980). A short-term study with chloroprene for the induction of lung tumors in Kunming Albino Mice with static

inhalation exposure was claimed positive (Dong et al. 1989).

An epidemiologic study on the carcinogenicity of chloroprene was negative (Pell 1978), but others were claimed by the authors to be positive (Khachatryan 1972a, b; Li et al. 1989). However, according to the IARC (1979), the studies of Pell and Khachatryan (1972a, b) have considerable methodological weaknesses; this also holds true for the study of Li et al. (1989).

Due to the inconsistency of these data, chloroprene was previously considered not classifiable as to its carcinogenicity to humans by the IARC (1987a). Our aim was to solve some of the contradictions of preceding studies by reinvestigating the mutagenicity of chloroprene in the Ames test, especially in the light of its limited chemical stability.

Materials and methods

Chemicals

2-Chloro-1,3-butadiene (CAS no: 126-99-8, lot no. 00020207; 50% in xylene): Johnson Matthey, Alfa Products, Karlsruhe, Germany; propylene oxide (99%), glutathione (GSH, reduced form), NADPH·NA × H_2O (nicotinamide adenine dinucleotide phosphate, reduced form, cell culture tested), Fluka, Neu-Ulm, Germany; Bacto Agar, Nutrient-Broth: Difco Laboratories, Detroit, Mich. USA; ampicillin sodium salt, *d*-biotin, β-D-glucose-6-phosphate sodium salt, L-histidin × H_2O (cell culture tested), benzo[a]pyrene, Sigma, St Louis, USA; DMSO (dimethyl sulphoxide, scintillation grade), xylene (p.A., 96%): Merck, Darmstadt, Germany; helium: CAS no. 7440-59-7, Messer Griesheim, Germany, crystal violet, hydroquinone (99%) phenothiazine (98%), *N,N*-dimethyl-4-nitrosoaniline (97%): Aldrich, Steinheim, Germany; S9 (from arochlor-1254-induced Sprague-Dawley rats, in sucrose), Organon Teknika, Durham, USA. Other chemicals were purchased in p.A. quality from Fluka, Neu Ulm, Germany.

Chloroprene was purchased as a solution in xylene (v.s.) and was distilled from this solution prior to the experiments: the distillation was done under nitrogen and normal pressure with a Liebig condenser. The fraction was collected with condensed at 59° C. Discarding of the first samples was not necessary, since chloroprene was the only low-boiling substance in the commercial solution.

The chloroprene distillates were stored in gas-tight head-space vials at –20° C under either O_2, argon or air. Aging of chloroprene was achieved by leaving the substance at room temperature (about 20° C). Chloroprene test solutions in ethanol or DMSO (0.5–0.025 M, dependent on the content of decomposition products) were prepared immediately before use. The distillates were always checked for purity by gas chromatography immediately after distillation and before preparing the test solutions, i.e. immediately before testing.

Instrumentation

Gas chromatography. Gas chromatograph: Shimadzu GC 8A (FID), CP Sil 5 CB column, Chrompack (50 m × 0.52 mm)

Gas chromatography-mass spectrometry (GC-MS). (a) Gas chromatograph: Hewlett-Packard HP 5890, KAS II: Gerstel; mass selective detector, 5970 A, Hewlett-Packard GmbH, column DB 624, J & W Scientific, (30 m × 0.32 mm).

(b) Gas chromatograph: Varian 3600, Darmstadt, Germany; column: PTE 5, Supelco, FRG (30 × 0.25 mm), detector: Ion-Trap Finnigan, Germany. For library search of spectra of known compounds the NIST library (National Institute for Science and Technology) was used.

GC, GC-MS conditions. For GC, liquid aliquots of 0.2 µl were injected. Injector/detector temperature: 270° C, oven temperature: 80° C. After 6 min the GC oven was heated to 220° C (heating rate 10° C/min). Carrier gas: nitrogen; flow rate: 8 ml/min. Integrator settings: attenuation: 4; slope: 500; stop time: 25 min.

Characterization of chloroprene byproducts was performed by GC-MS. For validation, GC-MS was done by two different methods, procedures (a) and (b).

Procedure (a): liquid aliquots of 0.2 or 0.5 µl chloroprene (1:25 in 1,1,1-trichloroethane) were injected "splitless" via a "cold-injection system" (KAS II: starting temperature: 35° C, heating rate: 10° C/s, final temperature: 220° C) on a DB 624 column. Oven temperature: 50° C, after 4.5 min heated to 260° C (heating rate 10° C/min), carrier gas: helium, detector temperature: 250° C, mass scanning 50–280 amu.

Procedure (b): liquid aliquots of 0.2 chloroprene were injected "splitless" via PTV (programmable temperature vaporizer: starting temperature: 60° C, heating rate: 300° C/min, final temperature: 250° C) on a PTE 5 column. Initial oven temperature: 60° C, heating rate: 10° C/min, final temperature: 250° C, carrier gas: helium, detection by Ion-Trap (electron impact mode), detector temperature: 200° C, mass scanning: 50–450 amu.

Ames test

The Ames test was performed according to Maron and Ames (1983) with the following variations: test mixtures [400 µl S9 mix or PBS (phosphate buffered saline), 100 µl TA 100 suspension, $1.5–2.5 \times 10^7$ cells, and 0–10 µl chloroprene as solution in DMSO or ethanol (p.A.), filled up with DMSO or ethanol to 10 µl] were preincubated at 37° C for 2 h, with gentle rolling (ca. 10 rpm) in gas-tight screw cap vials (2 ml, Hewlett-Packard). Before plating, the vials were left open until chloroprene had evaporated. This prevented contamination of the incubator, which could lead to high "spontaneous" revertant rates. For determination of toxicity, aliquots of a single dilution of the TA 100 cultures (1:800000 in nutrient broth) were incubated with the test compounds in the same manner as stated above, with the exception that these mixtures were plated on nutrient agar. Samples without chloroprene served for determination of cell density of the test cultures.

S. typhimurium TA 100 was kindly provided by D. M. Maron (Laboratory of B. N. Ames, University of California, Berkeley, USA). Spontaneous revertants ranged between 105 and 150 per plate.

Results

Pure chloroprene is unstable at room temperature and reacts rapidly with oxygen, sulphur, sulphur dioxide, etc. According to literature, it must be stored under an oxygen-free atmosphere, in the presence of inhibitors and/or below –15° C (Bauchwitz 1964). Chloroprene was purchased as commercial solution in xylene and therefore had to be distilled from this mixture before the experiments. The distillates were checked by GC immediately after distillation (Fig. 1a). One substance of the fresh distillate which was designated II in Fig. 1a could not be identified (see Discussion section). The triplet of peaks, designated III in Fig. 1a, represents the three xylene isomers. Two to 5% xylene remained in the chloroprene distillates (calculated on a molar basis).

Freshly prepared chloroprene, tested in ethanol solution as well as in DMSO, was not mutagenic in the Ames test with *S. typhimurium* TA 100 under the conditions described above, likewise with or without addition of S9 mix, additional NADPH or GSH. Under the same conditions, pro-

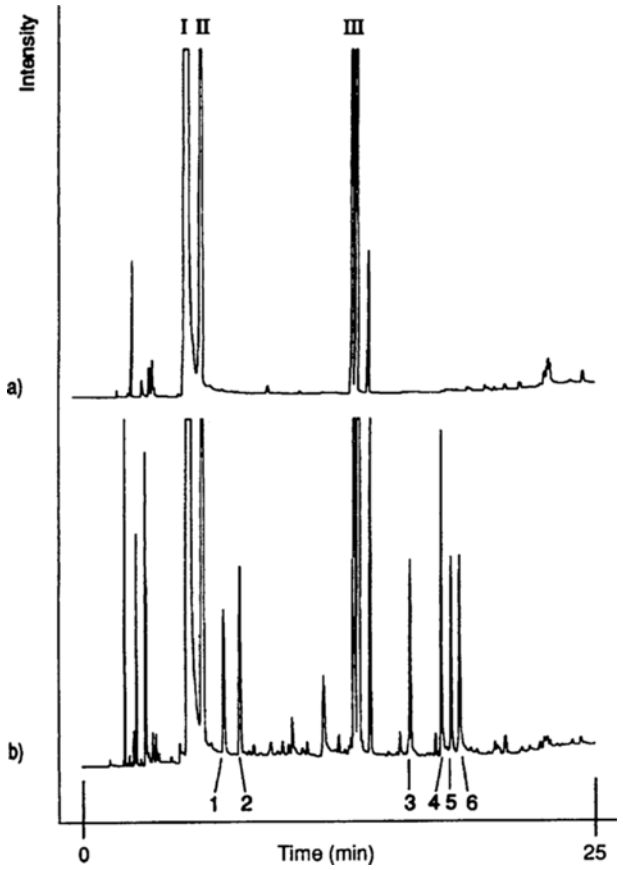


Fig. 1.a Gas-chromatogram of chloroprene, freshly distilled from a 50% solution in xylene. Peaks: *I*, chloroprene, *II*, not identified (probably 1-chloro-1,3-butadiene), *III*, xylene isomers. **b** Gas-chromatogram of aerobically aged (4 h at 20° C) chloroprene. Four of six (*1–6*) quickly emerging substances occur always in the same relative ratio (peaks designated *3,4,5,6*)

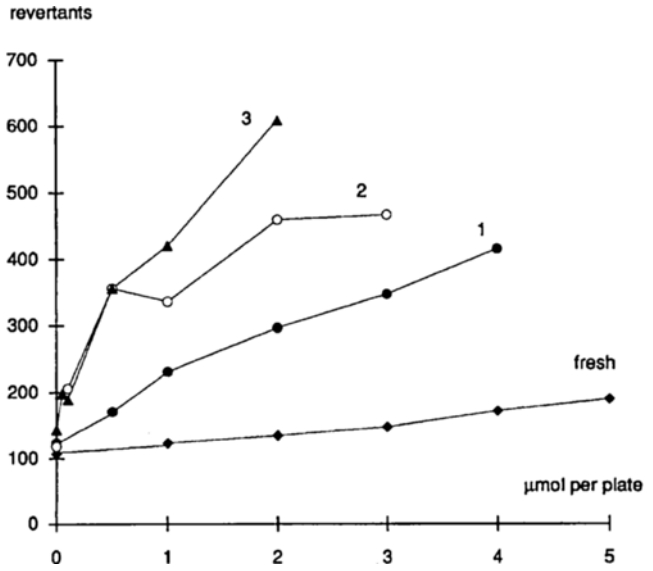


Fig. 2. Direct mutagenicity (revertants versus µmol per plate) of freshly distilled chloroprene ("fresh") towards *S. typhimurium* TA 100, in comparison to chloroprene which had been kept in 50 ml flask at –20° C for 1, 2 or 3 days (1, 2, 3). The distillations were performed on 4 successive days; the distillates were finally tested parallel at the same time

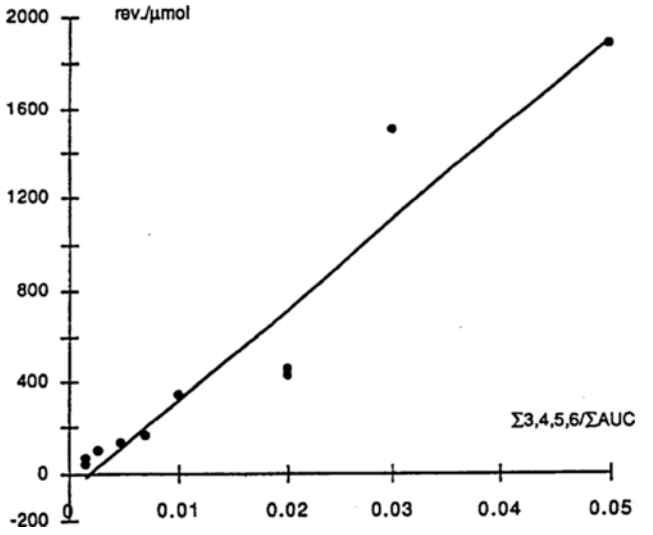


Fig. 3. Correlation of the cumulative amount of four "aerobic" chloroprene byproducts (peaks *3,4,5,6* of Fig. 1b) with the direct mutagenicity of aged chloroprene towards TA 100. Ten distillates with different portions of byproducts were tested in the Ames test, and the resulting mutagenicity (rev/µmol distillate) was plotted against the relative amount of byproducts *3,4,5,6* (Σ3,4,5,6 : ΣAUC, see text). The correlation coefficient is 0.96

pylene oxide (0.5 M in ethanol; 0–5 µmol per plate), used as positive control for a (volatile) direct mutagen, induced up to 550 revertants per plate; in agreement with Bootman et al. (1979). The activity of the S9 mix was checked using benzo[a]pyrene (0.25 M in DMSO); the highest dose tested (200 nmol per plate) induced 1065–1175 revertants per plate. Cytotoxicity was determined in parallel experiments: the survival rate at the highest concentrations tested was 15–30%, dependent on the solvent applied (ethanol or DMSO). Higher concentrations of chloroprene were 100% lethal.

A mutagenic effect occurred linearly with increasing age of the chloroprene distillates (Fig. 2). The mutagenicity of aged chloroprene was the same whether the preincubation was done in a gas-tight manner or not; toxicity was especially pronounced with a gas-tight preincubation.

Analysis by gas chromatography revealed decomposition products in aged chloroprene distillates (Fig. 1b, in comparison with Fig. 1a). The direct mutagenicity of aged distillates towards TA 100 correlated linearly with the cumulated amounts of four of these substances (Fig. 1b: peaks 3, 4, 5 and 6) which were always found in the same relative ratio to each other. For further proof, ten distillates of different ages containing different portions of decomposition products (according to GC analysis) were tested in the Ames test without S9. The resulting mutagenicity (revertants/µmol of aged chloroprene) of these distillates was correlated with the portion of the above mentioned decomposition products (3,4,5,6), relative to the whole distillate. For this calculation, the integrated peak areas of the products 3,4,5 and 6 (Σ3,4,5,6 in Fig. 3) were divided

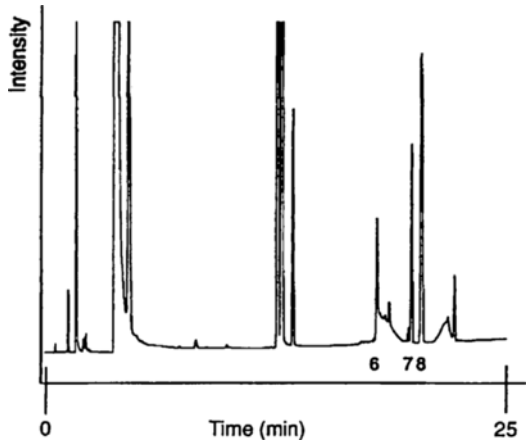


Fig. 4. Gas chromatogram of anaerobically aged chloroprene (distilled chloroprene, kept under argon for 48 h at 20° C). Peak *6* is identical with peak 6 in Fig. 1 b

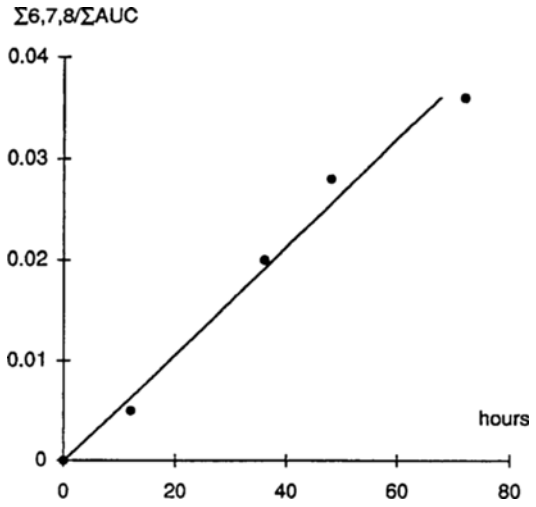


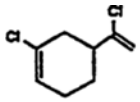
Fig. 5. Quantity of the "anaerobic" chloroprene decomposition products *6,7* and *8* (designation as in Fig. 4), expressed as the sum of these three compounds (Σ6,7,8), devided by the sum of all integrated peaks (ΣAUC), plotted against the incubation time (hours). The incubations were done at room temperature (about 20° C)

by the sum of all integrated peak areas (ΣAUC). Such a correlation could not be drawn for any of the other substances in aged chloroprene distillates.

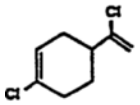
Glutathione at physiological concentrations (with and without S9 mix) reduced mutagenicity and cytotoxicity of aged chloroprene; the protective effect of GSH was less evident with increasing amounts of decomposition products (data not shown, see Westphal 1993).

In addition, the influence of the solvents was tested: chloroprene diluted in DMSO was markedly more toxic and more mutagenic than chloroprene dissolved in ethanol. To investigate the influence of the solvent xylene, 10% xylene (compared with chloroprene) was added, without any effect on the direct mutagenicity of aged chloroprene.

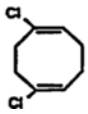
To investigate the conditions for formation of mutagenic decomposition products, distilled chloroprene was also aged anaerobically under argon: a different product pattern, parallel to aging of the chemical, was observed (Fig. 4).



1-Chloro-5-(1-chloroethenyl)-cyclohexene (CAS 13547-07-4)



1-Chloro-4-(1-chloroethenyl)-cyclohexene (CAS 13547-06-3)



1,6-Dichloro-1,5-cyclooctadiene (CAS 29480-42-0)

Aerobic incubation of chloroprene together with antioxidants (8 mg/ml hydroquinone, 2 mg/ml phenothiazine or 2 mg/ml *N,N*-dimethyl-4-nitrosoaniline) led to the same byproduct pattern as for chloroprene which had been incubated anaerobically under argon (data not shown). When chloroprene was kept at room temperature (about 20° C) under argon, significant amounts of the compounds designated 6,7 and 8 in Fig. 4 emerged within 12 h (Fig. 5). When chloroprene was kept under air (or O_2), detectable amounts of mutagenic decomposition products occurred within 30 minutes. In Fig. 1 b the gas chromatogram of a distillate is shown, which had been kept for 4 hours at 20° C. Additional substances occurred at later time points. No correlation of the bacterial mutagenicity with the quantities of substances appearing later could be detected.

Compound 6 of Fig. 1 b emerged on aerobic as well as on anaerobic aging, whereas at least two other peaks, designated 7 and 8 in Fig. 4, did not occur aerobically.

The direct mutagenicity of anaerobically aged chloroprene was only weak. Addition of S9 mix, however, led to an approximately two- to threefold increase in rate of mutations (up to 350 revertants per plate), dependent on the amount of anaerobic decomposition products and on the addition of NADPH.

Partial identification of compounds in aged chloroprene solutions (aerobic and anaerobic) was done by GC-MS: the solvent xylene and chloroprene itself were definitely identified.

In total, spectra of 15 decomposition products were recorded from aged chloroprene. Spectra library comparisons (see Material and methods section, instrumentation "b") concentrated on five aerobic and three anaerobic chloroprene decomposition products (including the common product no. 6 of Fig. 1 b and Fig. 4) for which structural proposals were made. These products displayed very similar mass spectra (e.g. m/z 53, 77, 88, 105, 141 and 176 amu). Such fragmentation patterns correspond with dimers of chloroprene. Proposed structures of such dimers are given in Fig. 6. These structures are consistent with those previously proposed by Stewart (1971). Because of the identical molecular weight of the compounds and their similar fragmentation, a positive assignment of these structures to individual peaks of Figs. 1 b and 4 could not be achieved. The remaining byproducts could not be related to reference spectra.

Discussion

One substance (designated II in Fig. 1 a) of the fresh distillate could not be identified. Since this substance elutes very close to chloroprene (compound I) and since its relative amount (ca. 2% calculated on the basis the integrated peak areas) did not vary significantly between different distillates, it most likely represents the isomer 1-chloro-1,3-butadiene which, according to the literature, is a normal byproduct of chloroprene. The amounts of this substance did not show any dependence on aging of the substance, or any correlation with the bacterial mutagenicity of aged chloroprene.

As mentioned initially, Bartsch et al. (1975, 1979) have reported on the mutagenicity of chloroprene in several *S. typhimurium* strains. The authors admitted that breakdown products or bacterial metabolism of chloroprene could have been responsible for at least part of the observed direct mutagenicity. However, they considered that dimers would not account for the mutagenicity of byproducts of chloroprene, because an (unspecified) mixture of cyclic dimers was found negative and "pure" chloroprene (99.7%) positive (Bartsch et al. 1975, 1979).

In the present experiments, freshly distilled chloroprene was not mutagenic. Obviously, decomposition products are responsible for mutagenic effects of aged chloroprene in *S. typhimurium*. Since the mutagenicity towards TA 100 was the same whether aged chloroprene was preincubated in a gas-tight manner or not, mutagenic byproducts of chloroprene ought to have a lower volatility than chloroprene, which by itself is strongly toxic to the bacteria.

There is reason to believe that the same or very similar decomposition products must have emerged in preceding experiments by other authors. This assumption is in line with applied test protocols: on testing chloroprene, Bartsch et al. (1975, 1979) calculated the weight of the liquid substance to give a defined final volume of vapour and placed the liquid in a desiccator which contained the test bacteria. The desiccator was partially evacuated, to allow vaporizing of the test compound. This was followed by introducing air, until atmospheric pressure was reached at 37° C. After 4 h exposure the test compound was removed. The formation of decomposition products from chloroprene by using this protocol is very likely (see Fig. 1 b, where chloroprene had been kept at 20° C for 4 h). Chloroprene was also positive in an Ames test by Willems (1978, 1980) who applied a similar test protocol, with the exception of exposing the bacteria for 24 and 48 h (the mutagenicity was more pronounced on 24-h incubation). In addition, the following compounds, derived from aged chloroprene solutions, were found positive: a purified "cyclobutane dimer" (designated LRT-315, structure not specified), purified "mixed intermediate isomers" (designated LRT-316) and a purified "cyclooctadiene isomer" (designated LRT-318, structure not specified), each with and without addition of S9. These dimers were tested without gas-tight preincubation (Willems 1978, 1980). These results are in line with the idea that dimers account for the mutagenicity of aged chloroprene.

Structures of chloroprene dimers were proposed by Stewart (1971). According to the literature (Bauchwitz 1964, Stewart 1971), dimerization of chloroprene proceeds in a Diels-Alder reaction. In the presence of free radical inhibitors, a mixture of vinylcyclohexene and cyclooctadiene compounds is formed in this way (Bauchwitz 1964).

In contrast to the preceding experiments (Bartsch et al. 1975, 1979; Willems 1978, 1980), liquid chloroprene was directly applied to the bacteria. It seems that, using this regimen, chloroprene decomposition products do not emerge in significant quantities, or are scavenged by other compounds in the incubation mixture.

According to the present results, a GSH-dependent detoxification became less effective with increasing amounts of byproducts (see Westphal 1993). This is in line with data of Nyström (1948) describing that aged chloroprene was more toxic for rats than the freshly prepared compound, and with Greim et al. (1981) who have put forward the idea that GSH might be involved in the detoxification of chloroprene.

Bartsch et al. (1975, 1979) reported on enhanced mutagenicity of chloroprene samples by addition of S9 mix. According to the present results, this effect might have been caused by reduced bacterial toxicity of chloroprene upon addition of S9 mix which could mimic metabolic activation. On the other hand, it seems that anaerobic byproducts may be activated dependent on NADPH.

From the present studies it is therefore obvious that assessments of the genotoxicity of chloroprene must consider the possibility of the formation of strongly mutagenic byproducts upon aging of the compound.

Acknowledgements. We thank Dr. E. Nolte and Dipl.-Ing. H. Meyer, Institut für Spektrochemie und angewandte Spektroskopie an der Universität Dortmund, for their practical advice.

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